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A Urease-Negative Mutant of *Helicobacter pylori* Constructed by Allelic Exchange Mutagenesis Lacks the Ability To Colonize the Nude Mouse Stomach

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The urease of *Helicobacter pylori* has been proposed to be one of its pathogenic factors. A kanamycin resistance determinant was inserted in a cloned urease gene, and transformation-mediated allelic exchange mutagenesis was carried out to introduce the disrupted gene into the corresponding wild-type chromosomal region of a clinical isolate of *H. pylori*, CPY3401. The resulting mutant, HPT73, had the null activity of urease. Nude mouse stomachs were challenged with these two isogenic strains to examine the role of urease in pathogenesis. Gastritis was found in the CPY3401-challenged stomachs, from which bacteria indistinguishable from CPY3401 were recovered. There was no gastritis in the HPT73-challenged stomachs, and we could not recover *H. pylori* from them. These results indicated that *H. pylori* urease is essential for colonizing the nude mouse stomach.

Helicobacter pylori is a pathogen for active chronic gastritis and is possibly involved in peptic ulceration (10). Several properties associated with *H. pylori* have been proposed to be pathogenic factors (6), among which is included the remarkably high level of urease activity. Involvement of urease in pathogenesis was first proposed by Eaton et al. (3), who demonstrated that the urease-deficient (Ure^-) mutant induced by a chemical mutagen could not colonize gnotobiotic piglets. Because of the chemical mutagenesis, it was difficult to rule out that their mutant acquired an undetected secondary mutation which affected colonizing ability. Another possibility that could not be excluded was that their mutant carried a pleiotropic mutation which led to lesions in production of other undefined pathogenic factors along with urease. The precise role of urease in pathogenesis can therefore be assessed unambiguously by construction of the genetically defined Ure^- mutant through molecular biological techniques. We previously developed an efficient genetic transformation system in *H. pylori* by the electroporation method, and a well-characterized kanamycin resistance (Km^r) gene inserted in a cloned *H. pylori* DNA could be incorporated into the recipient *H. pylori* genome by double-crossover-mediated homologous recombination (18). This property of the genetic exchange system has thus opened a way to carry out allelic exchange mutagenesis in *H. pylori*. Independently of our study, other groups (4, 7, 14, 15, 19) have recently reported similar phenomena, and two groups have succeeded in constructing the Ure^- and nonmotile mutants with this genetic exchange system (4, 7, 15). In this study, we constructed a Ure^- mutant by allelic exchange mutagenesis, and its pathogenesis was analyzed by using a nude mouse model that we had previously established (9).

H. pylori strains were, unless otherwise stated, cultivated at 37°C under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2) in brucella broth (Difco) medium supplemented with 5% fetal calf serum (Gibco BRL) or agar medium (1.5% agar) (18). For long-term storage, *H. pylori* strains were suspended in brucella broth containing 20% (wt/vol) glycerol and kept at −80°C.

In *H. pylori*, the nine genes (*ureA* to *ureI*) necessary for urease activity are clustered within an 8-kb region (2). The *ureA* and *ureB* genes encode the two subunits of urease, whereas the remaining genes are considered to encode accessory proteins. Plasmid pHP802 is a pACYC184 derivative carrying the *ureCDABIE* genes of *H. pylori* UMAB41 (Fig. 1) (8). A standard procedure for in vitro DNA manipulation (1) was employed to insert a 1.27-kb *Bam*HI-flanked Km^r gene of pUC4K (16) into the *Bam*HI site located at the *ureB* gene of pHP802. The resulting plasmid, designated pHPT54 (Fig. 1), was used as the donor DNA to transform a clinical isolate of *H. pylori*, CPY3401, which could colonize the nude mouse stomach (9). The electroporation method (18) gave rise to approximately 1.3×10^3 transformants per μg of DNA on agar plates containing 10 μg of kanamycin per ml, and all 12 of the transformants examined had the Ure^- phenotype on a rapid urease assay plate (urea agar base plate [BBL Microbiology Systems]). One such transformant, designated HPT73, was chosen for further study.

Southern analysis (1) was performed to determine the restriction maps of the CPY3401 and HPT73 *ure* regions. Total DNAs of the two strains were extracted by the established method (1), digested by *Bam*HI, *Hin*dIII, or *Bam*HI-*Hin*dIII, fractionated by electrophoresis in an agarose gel, and transferred to a nylon membrane. The DNA fragments used as the probes for the hybridization were the pUC4K-derived Km^r gene, the entire sequence of pACYC184 (12), and all of the *Bam*HI, *Hin*dIII, and *Bam*HI-*Hin*dIII fragments of pHP802 (Fig. 1), and they were labeled with digoxigenin-11-dUTP with the Boehringer Mannheim DIG labeling kit. A chemiluminescence detection kit (Boehringer Mannheim) was used to detect the chromosomal DNA fragments hybridized to each probe under low-stringency conditions, and some of the results are presented in Fig. 2. The physical map of the CPY3401 *ure* region exhibited some similarity to that of UMAB41 (Fig. 1).

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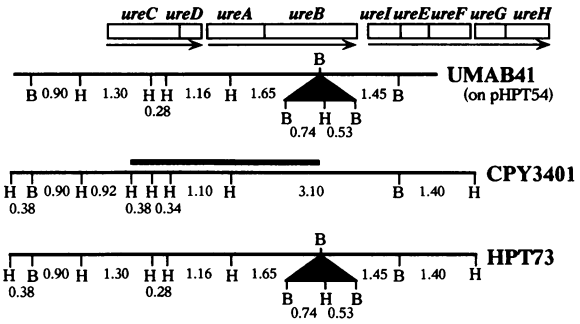

FIG. 1. Restriction maps of the *ure* regions of the three *H. pylori* strains UMAB41, CPY3401, and HPT73. Above the map of UMAB41 is depicted the organization of the urease genes; the arrows indicate the transcriptional direction of the operons deduced from the nucleotide sequence (2). The map of UMAB41 also represents that of pHPT54, a pHP802 derivative carrying a Km^r insert, indicated by a solid triangle (see reference 8 and this study). For simplicity, the vector region of pHPT54 is not depicted. The maps of CPY3401 and HPT73 were determined by Southern analyses in this study (see Fig. 2 and text). B, *Bam*HI; H, *Hin*dIII. The solid triangle indicates a pUC4K-derived Km^r gene (16). The numbers below each map indicate the length in kilobases between the restriction sites. The thick bar above the CPY3401 map indicates the minimum region replaced by the pHPT54-derived DNA to generate the HPT73 chromosome.

These two maps were discriminated in that (i) there was no *Bam*HI site in the *ureB* gene of CPY3401, (ii) the 1.30-kb *Hin*dIII fragment of UMAB41 covering the 5′ portion of the *ureC* gene was divided into two *Hin*dIII fragments (0.92 and 0.38 kb in size) in CPY3401, and (iii) the 1.44-kb segment of UMAB41 consisting of the 0.28- and 1.16-kb *Hin*dIII fragments was changed to a segment consisting of 0.34- and 1.10-kb *Hin*dIII fragments in CPY3401. This kind of restriction fragment length polymorphism in the *H. pylori ure* regions appears not to be unusual (4, 17). Subsequent Southern analysis indicated that the HPT73 *ure* region (i) lacked the pACYC184 sequence, (ii) possessed one copy of the Km^r determinant, and (iii) exhibited a physical map indistinguishable from that of the UMAB41-derived *ure* region on pHPT54 (Fig. 1 and 2). These results

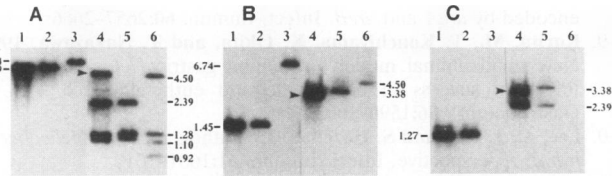

FIG. 2. Southern analyses of the CPY3401 and HPT73 *ure* regions. The probes used were the UMAB41-derived 5.29-kb *Bam*HI fragment of pHPT54 (i.e., the left-hand *Bam*HI fragment on pHPT54 in Fig. 1) (A), the UMAB41-derived 1.45-kb *Bam*HI fragment of pHPT54 (i.e., the right-hand *Bam*HI fragment on pHPT54) (B), and the pUC4K-derived 1.27-kb *Bam*HI fragment containing the Km^r gene (16) (C). The *Bam*HI-digested samples were loaded in lanes 1 to 3, and the *Hin*dIII-digested samples were loaded in lanes 4 to 6. The DNA samples loaded in lanes 1 and 4 were derived from pHPT54, those in lanes 2 and 5 were derived from HPT73, and those in lanes 3 and 6 were derived from CPY3401. The numbers to the sides refer to the sizes (in kilobases) of the fragments. The arrowheads in lane 4 indicate the fragments that consist of the *H. pylori* and vector DNA fragments. The hybridized fragments less than 0.38 kb in size could not be revealed in panel A.

indicated that HPT73 was formed by incorporation of the pHPT54-loaded Km^r gene and its flanking region into the CPY3401 chromosome by double-crossover-mediated homologous recombination. The minimum region of the recipient DNA inferred to be replaced by the donor DNA is depicted in Fig. 1.

The urease activity of CPY3401 cells grown in liquid medium was, according to the method of Ferrero and Lee (5), 16.39 ± 0.22 μmol of urea hydrolyzed per mg of total bacterial protein per min (n = 3), whereas that of HPT73 was not detectable (less than 0.03). The null activity of the HPT73 urease was consistent with the fact that the UreB subunit contains the catalytic site for the enzyme activity (8). The Ure$^-$ phenotype of HPT73 was stably maintained even after more than 30 passages on agar plates without addition of kanamycin. HPT73 showed no difference from CPY3401 regarding growth rate and motility in liquid medium. These results support the inference that HPT73 is isogenic to its parental strain, CPY3401, except for the *ure* region.

We previously established a model of *H. pylori*-associated gastritis by using a nude mouse stomach, and CPY3401 has been shown to colonize the gastric mucosa and cause chronic gastritis (9). This model system was used to assess the role of urease in pathogenesis. CPY3401 and HPT73 cells used for the challenge were derived from stocks at −80°C that had been prepared immediately after the cells' isolation or construction. The two strains were cultivated in the liquid media to late log phase to give rise to 10^8 cells per ml, and 1 ml of each strain was given as an oral challenge dose to the 6-week-old male BALB/c nude mice. One or 4 weeks after the challenge, the nude mice were sacrificed, and one-half of each stomach was subjected to histological study; the remaining half was, after homogenization, plated on Skirrow agar plates (Nissui, Tokyo, Japan) to recover *H. pylori*. The stomachs challenged with CPY3401 revealed, as described previously (9), superficial erosion and infiltration of mononuclear cells into the gastric mucosa and submucosal layer, whereas those challenged with strain HPT73 showed normal histology (Fig. 3). Consistent with the histological study, *H. pylori* could be recovered from the stomachs challenged with CPY3401 but not from those challenged with HPT73 (Table 1). The recovered *H. pylori* cells were indistinguishable from the challenge strain (CPY3401) in terms of urease activity, motility, and the physical map of the *ure* region. These results strongly suggested that *H. pylori* requires its urease to colonize the nude mouse stomach. Our previous work indicated that freshly isolated *H. pylori* strains, but not an established strain, NCTC 11637, could colonize the nude mouse stomach (9). Our later study has shown that CPY3401 exhibited infectivity even after (i) more than 50 passages by batch on Skirrow agar plates under growth conditions described previously (9) or (ii) storage for several months at −80°C (data not shown). It was therefore most probable that loss of the infectivity of HPT73 resulted from its *ure* mutation but not from another undefined mutation or mutations that occurred during genetic construction, purification, and storage at −80°C.

Two groups (11, 13) have demonstrated that, under the acidic conditions at pH 4 or below, (i) Ure$^+$ strains were more resistant in the presence of urea than in its absence and (ii) Ure$^-$ mutants could not survive. We also investigated the survival of CPY3401 and HPT73 after incubation for 1 h at 37°C in buffered solution at pH 2 as described by Pérez-Pérez et al. (11). CPY3401 cells survived at the level of 3.0×10^{-3} per input viable cell in the presence of 10 mM urea but could not survive ($<2.0 \times 10^{-7}$) in the absence of urea. The survival of HPT73 cells could not be detected ($<3.0 \times 10^{-7}$), regardless of whether urea was present in the buffer. Failure of

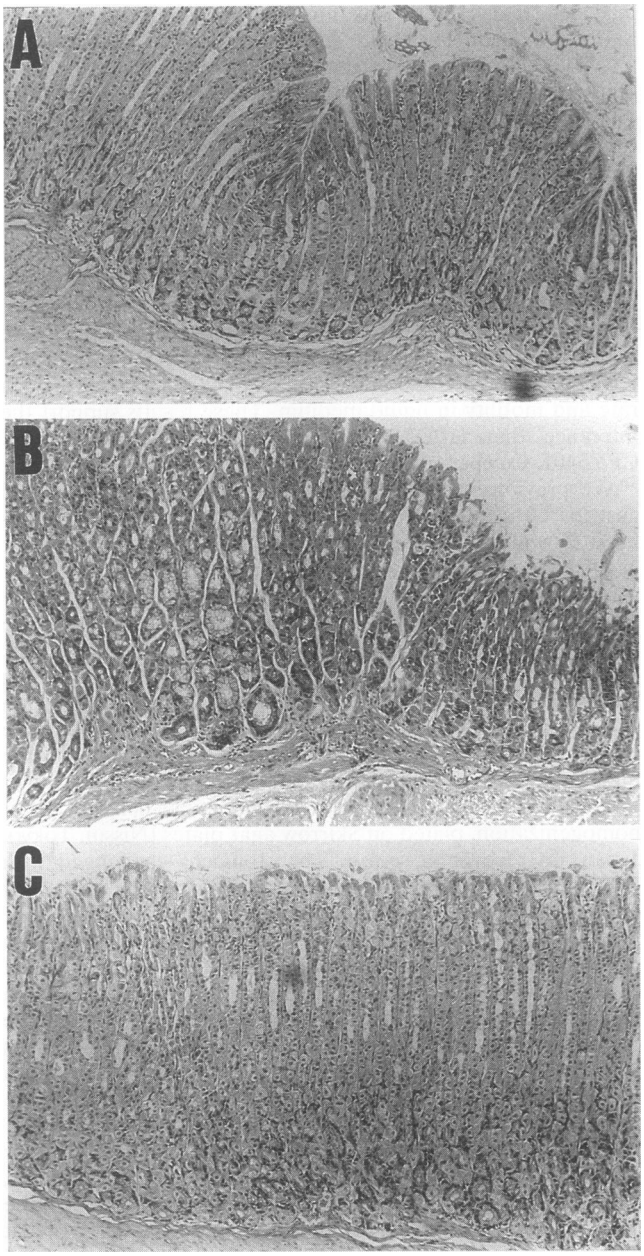

FIG. 3. Histological findings with nude mouse stomachs. Presented are the fundic areas of stomachs not challenged with *H. pylori* (A), stomachs 4 weeks after challenge with CPY3401 (B), and stomachs 4 weeks after challenge with HPT73 (C). Specimens were stained with hematoxylin-eosin. There were superficial gastric erosion and moderate infiltration of mononuclear cells in the gastric mucosa and submucosal layer in the CPY3401-challenged stomachs. Magnification, ×82.

HPT73 to colonize the nude mouse stomach is therefore probably ascribed to the inability to produce ammonia, which is able to neutralize the acidic milieu of the stomach. Such a consideration raises an interesting question as to whether HPT73 is able to colonize a stomach that is neutralized by treatment with the acid secretion inhibitors (e.g., proton pump inhibitors and H_2 receptor antagonists), and this kind of study is now in progress. If such a condition allows the colonization of HPT73, we may be able to assess unambiguously other proposed roles for ammonia produced by the action of urease (e.g., tissue inflammation and injury) (6).

TABLE 1. Recovery of *H. pylori* from nude mouse stomachs challenged with CPY3401 and HPT73

Expt[a]	Challenge strain	Challenge period (wk)	No. of colonies recovered[b]
1	CPY3401	1	2,948 ± 435
	HPT73	1	<5
2	CPY3401	4	3,336 ± 180
	HPT73	4	<5

[a] The detailed experimental protocol is described in the text. The two sets of experiments were carried out with different bacterial cultures.
[b] Values are means ± standard errors. Five mice were used in each experiment.

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